REMARKS

1. Based on the Office Action, the applicants have amended the claims to overcome the claim objection.

2. The application remarked that "the method taught by Caplan et al does not provide the bone marrow aspirate with a culture medium at the same time. Therefore, it would not be obvious to one of ordinary skill in the art to modify the method of isolating MSCs of Caplan et al." The Office Action stated that (Page 25, line 5 to line 9) "Caplan et al. teach providing mixture from bone marrow aspirate and combining the cell mixture with DMEM or complete medium." (e.g., column 8 line 45 to column 9 line 55 and column 45 line 45 to column 46 line 34) It should be stressed here that column 8 line 45 to column 9 line 55 cited are "specifically indicated below in Example 1" (column 8 line 20). Furthermore, the complete medium was "supplemented with serum" (column 8 line 48). The Example 1 did teach "aspirate of bone marrow (5-10ml) was transferred to sterile tubes to which 20ml complete medium was added" (column 19 line 47-48). However, there is no removing process or plate used to separate MSC taught in Example 1.

Example 6 of Caplan at al. (column 45 line 45 to column 46 line 34) did use Leukosorb to remove plasma and cleared of red blood cells. However, only DMEM was mentioned in Example 6. Caplan et al. used both DMEM and complete medium in Example 1, but only used DMEM in Example 6. In other words, the complete medium was taught by Caplan et al. to culture cells but was not utilized during the "removing red blood cells" process.

3. Caplan et al. used Leukosorb™ filter to remove fat, red blood cells and plasma. (column 46 line 14~15) However, the Leukosorb™ or its derivatives, which adsorb or trap leukocytes, can not remove leukocytes, as pointed by one patent (WO/2005/042784: DEVICE AND METHOD FOR HIGH-THROUGHPUT QUANTIFICATION OF MRNA FROM WHOLE BLOOD).

"Three anticoagulants were tested: ACD, EDTA, and heparin, with heparin resulting in the highest percent of leukocyte retention. While Leukosorb membranes have been used for ACD blood in transfusion, **approximately 15-40% of leukocytes passed through** even when four layers of membranes

were simultaneously used. EDTA blood was tested; the capacity and leukocyte retention was found to be similar to those for ACD. Most notably, however, **was that 100% of the leukocytes in heparin blood were trapped on the Leukosorb membranes**. The capture of 100% of leukocytes from heparin blood shows the reliability of quantification of mRNA using the present invention. These data indicate that the use of heparin blood is most suitable for the precise quantification of mRNA, whereas ACD blood is useful for applications requiring larger volumes of blood and less quantitative results." (cited the WO/2005/042784)

As indicated by Caplan et al., heparin was used (column 45 line 55-57, column 46 line 49). The percentage of leukocyte captured by Leukosorb™ will be 100%. Therefore, a monoclonal antibody separation is then needed to separate MSCs. (column 46 line 35~61)

The Leukosorb™ or its derivatives trap leukocytes. However, on the opposite, the application used upper plate with pores to remove small-sized cells such as leukocyte. The Leukosorb™ traps leukocytes would teach away this application to remove leukocytes. Therefore, it would have not been obvious to one of ordinary skill in the art at the same time the upper plate taught by this application was made by modification from the Leukosorb™ used by Caplan et al.

4. As indicated in MPEP 706.02(j) (Contents of a 35 U.S.C. 103 Rejection [R-6] - 700 Examination of Applications): To support the conclusion that the claimed invention is directed to obvious subject matter, either the references must expressly or impliedly suggest the claimed invention or the examiner must present a convincing line of reasoning as to why the artisan would have found the claimed invention to have been obvious in light of the teachings of the references." *Ex parte Clapp*, 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985).

Caplan et al. has disclosed Leukosorb™ to remove plasma and cleared of red blood cells and the method of culturing MSC. Was there any problem existed in the disclosure of Caplan et al.? Otherwise, how come a person with ordinary skill in the art wants to combine the teachings of Caplan et al. and Rieser et al.? In other words, the obvious rejection did not "present a convincing line of reasoning as to **why** the artisan would have found the claimed invention to have been obvious in light of the teachings of the references."

5. As the Office Action indicated, Rieser et al teach that bone substitute plate (7) serves two functions: it is a permeable wall for the cell space (1), and it provide a substrate for adherence of cells. Moreover the abstract of Rieser et al clearly indicated that "The cells settle on such a plate (7) and the cartilage tissue growing in the cell space (1) **grows into pores or surface roughness of the plate**, whereby an implant forms which consists of a bone substitute plate (7) and a cartilage layer covering the plate and whereby the two implant parts are connected to each other in positively engaged manner by being grown together."

How could one with ordinary skill in the art want to combine the teaching of Rieser et al. with that of Caplan et al? Especially, the bone substitute plate of Rieser et al. is used as a filter. Furthermore, Rieser et al discredited the filter material in US Pat. No 5326357. (column 2 lines 42-46) **It would be very illogical** that one with ordinary skill in the art read Rieser's teaching and then used the element functioning as a filter as they disbelieved.

6. Rieser et al also claim MSCs to grow into pores or surface roughness of the plate (claim 14). The US patent 5634879 also stated that "it is believed that this roughened surface exposes a greater surface anchoring area to cells for attachment." (column 6, lines 2-4) Even thought the plate taught by Rieser et al. functions as a filter, the result of isolating efficiency is still far behind from this application because that the surface roughness of bone substitute exposes a greater surface anchoring area to cells for attachment. Besides, the plate taught by Rieser et al. is made of hydroxyapatite. Hydroxyapatite (calcium phosphate) is the inorganic matrix of bone tissues. MSCs when cultured in expansion medium without any osteogenic differentiation additives will develop to osteoblast (Abstract, in Journal of Cellular and Molecular Medicine 12:281-291, 2008). Because bone cells such as osteoblasts will lay down osteoid (contains calcium phosphate) and transform into osteocytes embedded in mineralized bone matrix (Abstract, in Dev Dyn. 235:176-190, 2006), MSCs cultured on plate used by Rieser et al will differentiate into osteoblasts and buried themselves in mineralized bone matrix such as hydroxyapatite even in the medium used for expansion. Consequently, the differentiated MSCs will not maintain as undifferentiated cells and will be difficult for isolation. Therefore, the difference between this application and the prior arts cited would not be obvious.

"Indeed, cells on calcium phosphate without osteogenic differentiation additives developed to osteoblasts as shown by increased ALP activity and expression of

osteogenic genes, which was not the case on tissue culture plastic." (Cited in Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. Journal of Cellular and Molecular Medicine 12:281-291, 2008)

"During osteogenesis, osteoblasts lay down osteoid and transform into osteocytes embedded in mineralized bone matrix." (Cited in Buried alive: how osteoblasts become osteocytes. Dev Dyn. 235:176-190, 2006)

7. Regarding the obvious rejection over Caplan et al. in view of Prockop et al. and Mastui et al., the Office Action indicated that " Caplan et al. do not teach the method of isolating MSCs where the mixed population of cells in medium is seeded into a culture device..." However, Caplan et al. do teach other methods to isolate and culture MSCs. Without the blueprint of this application, how can one with ordinate skill in the art want to combine all of teachings of Caplan et al., Prockop et al. and Mastui et al.? Especially, the membranes filter 2 of Mastui et al. is not the same as the upper plate of this application, which is made of made of the mesenchymal stem cell adhering material. Furthermore, Prockop et al. teach the separation of RS (recycling stem cells) from non-RS mMSCs, but this application separates MSCs from the other small-sized cells.

Again, there is no reason "**why** the artisan would have found the claimed invention to have been obvious in light of the teachings of the references.", as the MPEP 706.02(j) indicated.

In sum, not only the steps of this application are different from the prior arts, but also there are no reasons to find this application in light of the teachings of the references. Accordingly, this application should be placed in condition of allowance. An early Notice to this effect is respectfully expected.

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Buried Alive: How Osteoblasts Become Osteocytes

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During osteogenesis, osteoblasts lay down osteoid and transform into osteocytes embedded in mineralized bone matrix. Despite the fact that osteocytes are the most abundant cellular component of bone, little is known about the process of osteoblast-to-osteocyte transformation. What is known is that osteoblasts undergo a number of changes during this transformation, yet retain their connections to preosteoblasts and osteocytes. This review explores the osteoblast-to-osteocyte transformation during intramembranous ossification from both morphological and molecular perspectives. We investigate how these data support five schemes that describe how an osteoblast could become entrapped in the bone matrix (in mammals) and suggest one of the five scenarios that best fits as a model. Those osteoblasts on the bone surface that are destined for burial and destined to become osteocytes slow down matrix production compared to neighbouring osteoblasts, which continue to produce bone matrix. That is, cells that continue to produce matrix actively bury cells producing less or no new bone matrix (passive burial). We summarize which morphological and molecular changes could be used as characters (or markers) to follow the transformation process. *Developmental Dynamics 235:176–190, 2006.* © 2005 Wiley-Liss, Inc.

Key words: osteoblast; osteocyte; osteogenesis; intramembranous ossification; preosteoblast; osteoid-osteocyte; preosteocyte

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INTRODUCTION

It has been known, for almost one and a half centuries, that osteocytes are derived from osteoblasts (Gegenbauer, 1864). Osteoblasts (bone forming cells) are of mesenchymal origin, secrete non-mineralized bone matrix (osteoid), and finally become incorporated as osteocytes in mineralized bone matrix. Osteocytes are by far the most abundant cellular component of mammalian bones, making up 95% of all bone cells (20,000 to 80,000 cells per mm^3 bone tissue) that cover 94% of all bone surfaces (Frost, 1960; Marotti, 1996); there are approximately ten times more osteocytes than osteoblasts in an individual bone (Parfitt, 1990). In humans, osteocytes can live long. Frost (1963) estimates the average half-life of a human osteocyte as 25 years. However, when we consider an overall bone-remodelling rate of between 4 to 10% per year (Delling and Vogel, 1992; Manolagas, 2000), the life of many osteocytes may be shorter (Marotti et al., 1990). Furthermore, the lifespan of osteocytes greatly exceeds that of active osteoblasts, which is estimated to be only three months in human bones (Manolagas, 2000) and 10–20 days in mouse alveolar bone (McCulloch and Heersche, 1988). Osteocytes communicate with one another and with cells at the bone surface via a meshwork of cell processes that run through canaliculi in the bone matrix (Palumbo et al., 1990). Thus, bone cells form a functional network within which cells at all stages of bone formation from preosteoblast to mature osteocyte remain connected.

The literature provides us with an astounding number of terms concerning the transition from osteoblast to osteocyte, such as "osteocytes are *encased* in mineralized matrix" (Holtrop,

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Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells

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Abstract

Although studies *in vivo* revealed promising results in bone regeneration after implantation of scaffolds together with osteogenic progenitor cells, basic questions remain how material surfaces control the biology of mesenchymal stem cells (MSC). We used human MSC derived from bone marrow and studied the osteogenic differentiation on calcium phosphate surfaces. In osteogenic differentiation medium MSC differentiated to osteoblasts on hydroxyapatite and BONITmatrix®, a degradable xerogel composite, within 14 days. Cells revealed a higher alkaline phosphatase (ALP) activity and increased RNA expression of collagen I and osteocalcin using real-time RT-PCR compared with cells on tissue culture plastic. To test whether material surface characteristics alone are able to stimulate osteogenic differentiation, MSC were cultured on the materials in expansion medium without soluble additives for osteogenic differentiation. Indeed, cells on calcium phosphate without osteogenic differentiation additives developed to osteoblasts as shown by increased ALP activity and expression of osteogenic genes, which was not the case on tissue culture plastic. Because we reasoned that the stimulating effect on osteogenesis by calcium phosphate surfaces depends on an altered cell–extracellular matrix interaction we studied the dynamic behaviour of focal adhesions using cells transfected with GFP labelled vinculin. On BONITmatrix®, an increased mobility of focal adhesions was observed compared with cells on tissue culture plastic. In conclusion, calcium phosphate surfaces are able to drive MSC to osteoblasts in the absence of osteogenic differentiation supplements in the medium. An altered dynamic behaviour of focal adhesions on calcium phosphate surfaces might be involved in the molecular mechanisms which promote osteogenic differentiation.

Keywords: mesenchymal stem cells • calcium phosphate surface • focal adhesion • osteoblast

Introduction

Tissue engineering of bone based on the combination of multi-potent mesenchymal stem cells (MSC) and a scaffold represents a new promising approach in bone regeneration to restore bony tissue after extensive loss due to injury or disease [1–5]. Although studies *in vivo*, both animal experiments and clinical trials have shown the usefulness of the implantation of scaffolds with osteogenic progenitor cells to regenerate bone, a number of basic questions remain before application of tissue engineering

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